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Relationship between pH and Medium Dissolved Solids in Terms of Growth and Metabolism of Lactobacilli and *Saccharomyces cerevisiae* during Ethanol Production

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The specific growth rates of four species of lactobacilli decreased linearly with increases in the concentration of dissolved solids (sugars) in liquid growth medium. This was most likely due to the osmotic stress exerted by the sugars on the bacteria. The reduction in growth rates corresponded to decreased lactic acid production. Medium pH was another factor studied. As the medium pH decreased from 5.5 to 4.0, there was a reduction in the specific growth rate of lactobacilli and a corresponding decrease in the lactic acid produced. In contrast, medium pH did not have any significant effect on the specific growth rate of yeast at any particular concentration of dissolved solids in the medium. However, medium pH had a significant ($P < 0.001$) effect on ethanol production. A medium pH of 5.5 resulted in maximal ethanol production in all media with different concentrations of dissolved solids. When the data were analyzed as a 4 (pH levels) by 4 (concentrations of dissolved solids) factorial experiment, there was no synergistic effect ($P > 0.2923$) observed between pH of the medium and concentration of dissolved solids of the medium in reducing bacterial growth and metabolism. The data suggest that reduction of initial medium pH to 4.0 for the control of lactobacilli during ethanol production is not a good practice as there is a reduction ($P < 0.001$) in the ethanol produced by the yeast at pH 4.0. Setting the mash (medium) with ≥30% (wt/vol) dissolved solids at a pH of 5.0 to 5.5 will minimize the effects of bacterial contamination and maximize ethanol production by yeast.

Lactic acid bacteria and yeasts are often encountered together in natural ecosystems and are known to compete for the same nutrients (1). The occurrence of these lactic bacteria during ethanol production by yeast is therefore the primary cause for a variety of yield and productivity problems (2, 3, 5, 7, 8, 10, 16, 17, 18). Growth of lactic acid bacteria generates acidic fermentation end products that accumulate in the extracellular environment. The pronounced organic acid production by these bacteria reduces the pH of the medium, which creates an unfavorable environment for many other organisms, yeast included.

In fuel ethanol distilleries, reduction in lactic acid bacterial contamination is currently achieved by using antibiotics. The extensive use of antibiotics such as penicillin G over the last 50 years has led to the emergence of resistant microflora (18). Therefore, other antibiotics used in the animal feed industry, such as virginiamycin (11) and monensin (20), have been introduced into the alcohol industry. However, the concept of antibiotic use in an industrial process is undesirable, despite the absence of antibiotic residues in spent grains, which are destined to be fed to livestock. The misuse of antibiotics contributes to the development of reservoirs of resistant bacteria. Genes encoding resistance to tetracycline in the microflora of watercourses near swine production facilities that used tetracycline as a growth promotional antibiotic have been reported (4). Lima et al., (15) found 86% and 43% of bacteria isolated from agricultural environments to be resistant to 100 U penicillin/ml and 300 μg/ml virginiamycin, respectively. There is therefore a need in the ethanol industry to search for alternatives to the use of antibiotics in the control of bacterial contamination. One approach would be to totally eliminate the use of antibiotics during ethanol production. This can be achieved by changing the process conditions in such a way that the bacterial growth rate is reduced significantly while the yeast outgrows the bacteria and produces ethanol. In fuel ethanol plants that practice continuous fermentation, lowering the pH of the mash to 4.0 or less is one of the practices routinely followed to control contaminating bacteria.

Other than reducing the pH of the medium, one possible way to control lactobacilli may be to increase the osmotic stress placed upon these bacteria by increasing the concentration of dissolved solids in the medium. Concentrations of dissolved solids of 20 to 24 g per 100 g grain mash are considered normal gravity by beverage and fuel alcohol distillers (22). Very-high-gravity (VHG) fermentation technology has been defined as preparation of mash containing 300 or more grams dissolved solids per liter mash (21). Subsequent to this definition of VHG technology, a patent was issued for alcohol production by fermentation of "high dry solids mash" (14). Among the number of advantages listed by Thomas et al. (22) for VHG fermentation technology are reduced survival and proliferation of contaminating bacteria. The present study was therefore designed to assess the relationship between pH of the medium and concentration of dissolved solids in the medium on the growth and metabolism of lactobacilli and yeast.

MATERIALS AND METHODS

Microorganisms. An industrial strain of *Saccharomyces cerevisiae* (Allyeast Superstart; Alltech, Inc.) was used. *Lactobacillus plantarum*, *L. paracasei* subsp. *paracasei*, *L. pentosus*, and *L. casei* subsp. *rhamnosus* (ATCC 21052) were the

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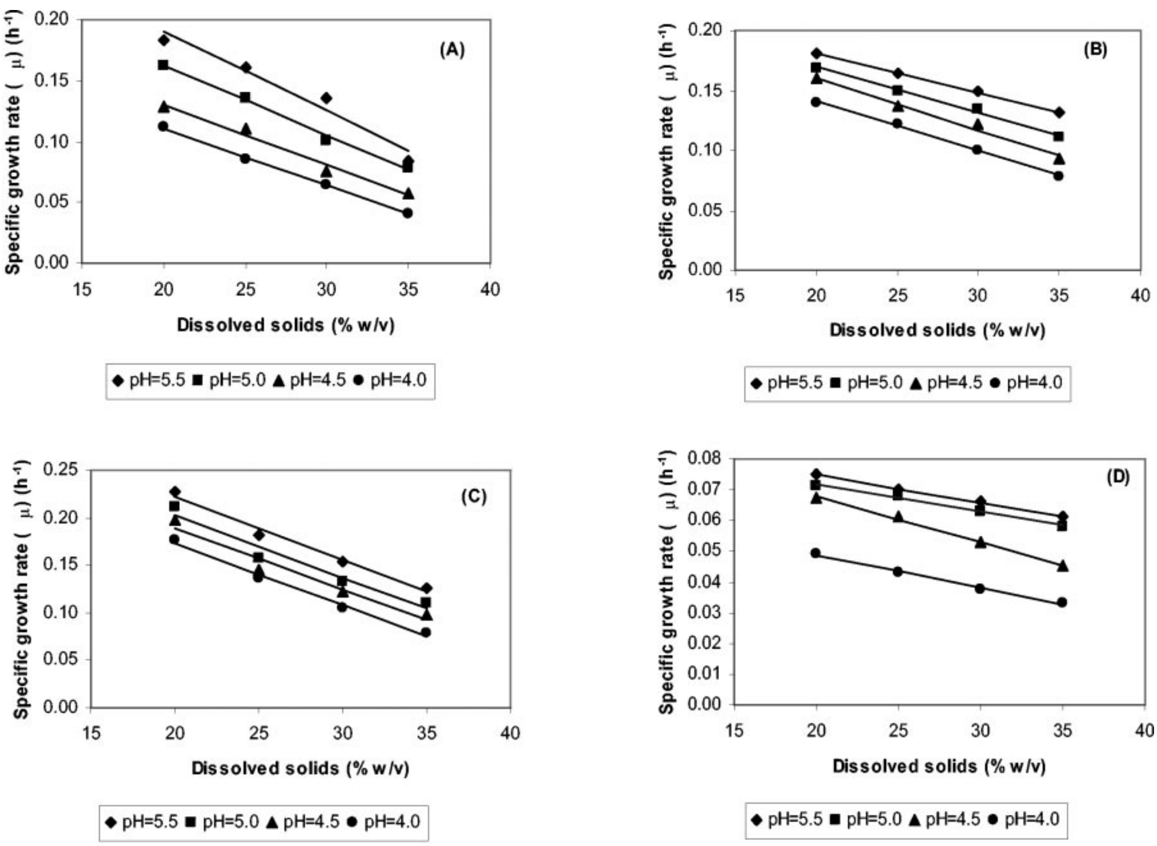

FIG. 1. Specific growth rates (μ) of various lactobacilli at 30°C: (A) *L. plantarum*, (B) *L. paracasei*, (C) *L. pentosus*, and (D) *L. rhamnosus.* Bacteria were grown in media with different concentrations of dissolved solids (maltodextrin concentration) at four pH levels.

bacteria used. The non-ATCC strains were isolated from contaminated mash samples obtained from two different fuel ethanol distilleries and were identified by both the API CHL50 and Biolog Microlog microbial identification systems.

Initial concentrations of dissolved solids chosen. A variety of concentrations of dissolved solids were chosen to cover the range extending from normal gravity to very-high-gravity fermentations.

Growth media used. Yeast extract-peptone-dextrose medium was used. The dextrose portion of the medium was substituted with maltodextrins at the concentrations chosen. Medium was made up and autoclaved at 121°C for 20 min. Four sets of media were made for each maltodextrin concentration, and pH for each set was adjusted using 85% *o*-phosphoric acid. The different pH values chosen were 4.0, 4.5, 5.0, and 5.5, which are the pH values most commonly encountered in the industry.

Determination of microbial growth and metabolism. Frozen cultures of all the bacteria listed for the study were inoculated into fresh sterile MRS broth (50 ml) in 100-ml dilution bottles. The cultures were grown for 21 h at 30°C on an orbital shaker (200 rpm). Similarly, the yeast was grown in YM broth. After 21 h, growth of suitable dilutions of the cultures was measured as optical density at a wavelength of 600 nm (OD_{600}). Appropriate dilutions were made in fresh sterile growth media (media with different levels of dissolved solids) to achieve an initial inoculum of $\sim 1 \times 10^8$ cells/ml (0.1 OD_{600} unit = $\sim 1 \times 10^8$ cells/ml for bacteria). Filter-sterilized Allcoholase II L400 (amyloglucosidase) was added to the different media at 0.06% (wt) of dissolved solids just before the addition of bacteria or yeast. The cultures were added to sterile 96-well flat-bottom microtiter plates (Fisher Scientific). A quantity of 100 μl of culture was added to each well to yield 10^7 cells per well. This number was chosen since there has to be at least 10^7 cells/ml of contaminating bacteria in the mash to cause a significant reduction in ethanol production (17). Appropriate blanks with 100 μl of uninoculated media were also included. The plate was inoculated aseptically and was sealed with a sterile film (Breathe Easy sealing membrane; Diversified Biotech, Boston, MA). Plates were then incubated in a plate reader (iEMS reader MF; MTX Labsystems, Inc., Vienna, VA) at 30°C. Bacterial growth as OD_{600} was measured every 2 h for 48 h using the Ascent software v.2.6 (MTX Labsystems, Inc.). Bacterial and yeast growth curves were generated using the data obtained from which specific growth rates (μ in h^{-1}) were calculated.

Suitable dilutions of the overnight cultures of the organisms (to obtain an initial count of $\sim 10^7$ cells/ml) were performed in 250-ml sterile Erlenmeyer flasks containing 50 ml of respective media. These flasks were incubated at 30°C for 48 h in an orbital shaker (175 rpm). Samples were withdrawn after 48 h and filtered through a 0.2-μm filter, and the filtrate was analyzed for lactic acid production (in the case of bacteria) and ethanol production (in case of yeast) using high-performance liquid chromatography (HPLC). Analysis for each organism at each concentration of dissolved solids and at each pH was performed in duplicate.

HPLC analysis. Sugars and lactic acid were quantitated by HPLC analysis. A 20-μl aliquot from a suitably diluted fermentation sample was subjected to chromatography on an HPX-87H column (Bio-Rad Laboratories, Hercules, CA), maintained at 65°C, which analyzes sugars, alcohols, and organic acids. Sulfuric acid (2 mM) was used as the mobile phase at a flow rate of 0.6 ml/min. The components were detected with a differential refractometer (model 2410; Waters Chromatographic Division, Milford, MA). The data were processed using the Millennium[32] computer program (Waters Chromatographic Division).

RESULTS

Growth rate of bacteria. The specific growth rates of all four species of lactobacilli decreased linearly ($r^2 > 0.97$) as the concentration of dissolved solids increased in the medium (Fig. 1). The growth rates successively declined as the pH of the medium decreased.

Lactic acid production by the bacteria. The reduction in growth rates was directly correlated to decreased lactic acid production by the lactobacilli tested. As the concentration of

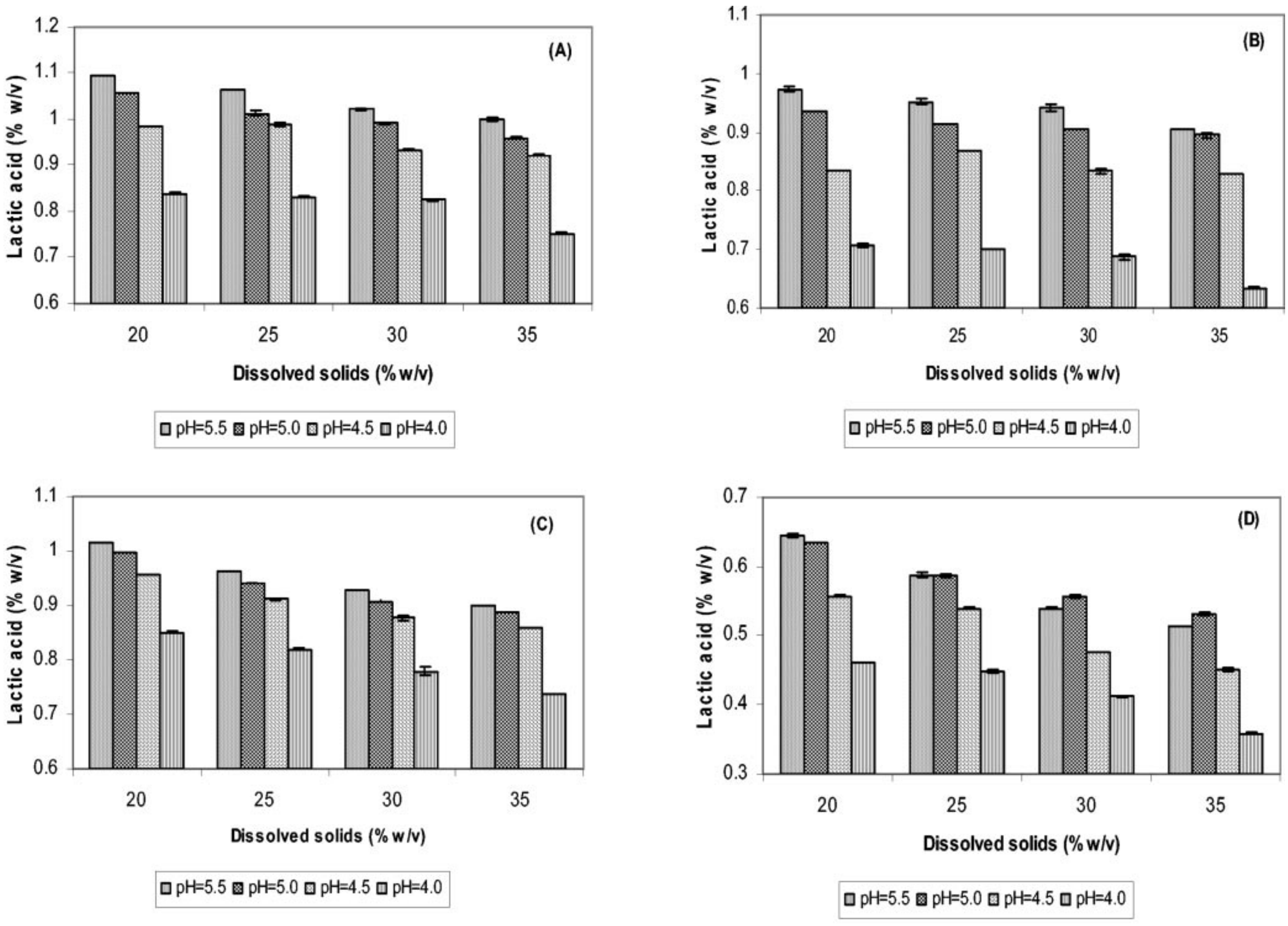

FIG. 2. Lactic acid produced by various lactobacilli after 48 h at 30°C: (A) *L. plantarum*, (B) *L. paracasei*, (C) *L. pentosus*, and (D) *L. rhamnosus*. Bacteria were grown in media with different concentrations of dissolved solids (maltodextrin concentration) at four pH levels. Error bars indicate ± standard deviation.

dissolved solids in the medium increased, the lactic acid production decreased at each pH level studied. As the pH of the medium decreased, the lactic acid production by the bacteria also decreased ($P < 0.001$) (Fig. 2). The data obtained were analyzed as a 4 (pH levels) by 4 (concentration of dissolved solids) factorial experiment, using SAS software. There was no synergy ($P > 0.2923$) observed between pH and concentration of dissolved solids in inhibiting the lactobacilli.

Growth rate of yeast and ethanol production. In contrast to bacteria, the specific growth rate of yeast increased as the concentration of dissolved solids in the medium increased. The pH of the medium had no significant effect on the specific growth rate at any particular concentration of dissolved solids in the medium (Fig. 3). However, medium pH had a significant impact on the final ethanol concentration in the medium. A reduction ($P < 0.001$) in the final ethanol produced was observed as the pH of the medium reduced. However, an increase in final ethanol concentration was observed as the concentration of dissolved solids (sugars) in the medium increased (Fig. 4). For a given concentration of dissolved solids in the medium, more residual sugars were present after 48 h of fermentation at a pH of 4.0 than at a pH of 5.5 (Fig. 5).

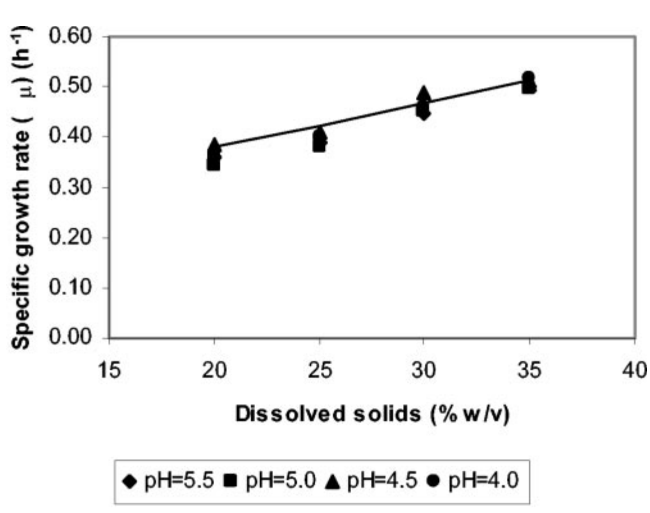

FIG. 3. Specific growth rate (μ) of *Saccharomyces cerevisiae* at 30°C in media with different concentrations of dissolved solids (maltodextrin concentration) at four pH levels.

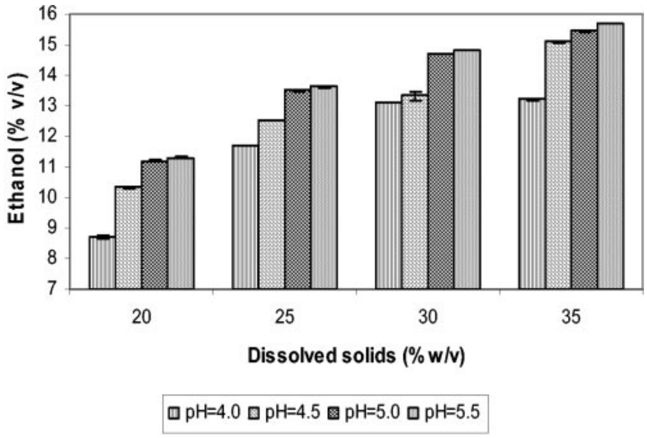

FIG. 4. Ethanol produced by *S. cerevisiae* in 48 h at 30°C with different concentrations of dissolved solids (maltodextrin concentration) in the medium at four pH levels. Error bars indicate ± standard deviation. The coefficient of variation among the duplicates was <2%.

DISCUSSION

The results clearly demonstrate that the growth rate of lactobacilli is reduced considerably when there is an increase in the concentration of dissolved solids in the medium. The higher sugar concentration in the medium most likely exerts a severe osmotic stress on the bacterial cells. For active metabolism to occur, the intracellular conditions must remain relatively constant with respect to ionic composition, pH, and metabolite levels (6). A change in the osmolality of the environment could, therefore, rapidly compromise essential cell functions, and bacteria need to adapt to such a change in their environment to survive. In general, the bacteria can adapt to such changes by accumulating compatible solutes (by uptake or synthesis) under hyperosmotic conditions and releasing (or degrading) them under hypoosmotic conditions. A rapid response to changing osmotic conditions has been studied in detail in *Lactobacillus plantarum* and *Lactococcus lactis* by Glaasker et al. (9). However, these authors reported a stimulatory effect of glycine-betaine, a compatible solute, only in the case of salt stress (KCl or NaCl).

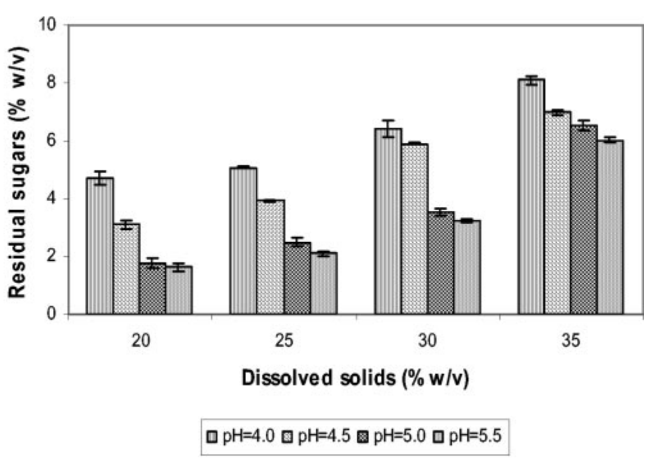

FIG. 5. Residual sugars in the medium (total of dextrins, maltotriose, maltose, and glucose) after fermentation at four pH levels by *S. cerevisiae* for 48 h at 30°C. Error bars indicate ± standard deviation. The coefficient of variation among the duplicates was <5%.

Because *Saccharomyces cerevisiae* can ferment mashes with up to 38% (wt/vol) dissolved solids under the right conditions (21), utilizing fermentation mashes with 30% or more dissolved solids may help to provide the right conditions for yeast to outcompete contaminating bacteria. This practice may help to minimize losses in ethanol yield due to bacterial contamination. Fuel ethanol distilleries currently use corn mashes containing up to 30% dry solids. However, the content of dissolved solids is not measured after mashing. Based on the liquefaction procedure followed, the content of dissolved solids of mashes could be significantly less than 30% (wt/vol), while the plant has a mash with 30% dry solids.

With respect to medium pH, except for some species of *Lactobacillus* and *Leuconostoc*, lactic acid bacteria are neutrophiles: i.e., their optimal pH for growth lies between 5 and 9 (12). Lower extracellular pH is harmful to these bacteria. This is attributed to acidification of cytoplasmic pH below a threshold value and subsequent inhibition of cellular functions (13). In general, *Saccharomyces cerevisiae* is an acidophilic organism and, as such, grows better under acidic conditions. The optimal pH range for yeast growth can vary from pH 4 to 6, depending on temperature, the presence of oxygen, and the strain of yeast. There is a paucity of literature on the physiological basis for this preferred pH range, but it most likely is due to the optimum pH value for the activity of plasma membrane-bound proteins, including enzymes and transport proteins.

During growth, it is important for the yeast to maintain a constant intracellular pH. There are many enzymes functioning within the yeast cell during growth and metabolism. Each enzyme works best at its optimal pH, which is acidic because of the acidophilic nature of the yeast itself. When the extracellular pH deviates from the optimal level, the yeast cell needs to invest energy to either pump in or pump out hydrogen ions in order to maintain the optimal intracellular pH (19, 23). If the extracellular pH deviates too much from the optimal range, it may become too difficult for the cell to maintain constant intracellular pH, and the enzymes may not function normally. If the enzymes are deactivated, the yeast cell will not be able to grow and make ethanol efficiently. That is the most likely explanation for the observed reduction in ethanol production when the initial medium pH was lowered from 5.5 to 4.0 (Fig. 4). This also resulted in increased residual sugars at the lower pH (Fig. 5). The pH of the medium did not affect the saccharification process, since normal glucose production was observed even in the media set at a pH of 4.0. Similar observations with the saccharification activity of glucoamylase were observed in previous studies (17).

Raising the external pH closer to the desired intracellular pH places less stress on cells, which results in lower energy wastage to maintain the internal pH within a range optimal for growth. In particular, in media containing organic acids, raising the pH to a value higher than the pK_a value of the acid reduces the concentration of undissociated acid for a given amount of total acid. This results in a decrease in the inhibitory effect of the organic acid on yeast growth and metabolism. At higher pH values, the difference between the internal and external pH values (ΔpH) is smaller. This could be a reason for reduced inhibition of yeast growth at higher pH since the accumulation of undissociated acids within the cell is a function of ΔpH.

The results presented in this paper suggest that lowering the initial pH of the medium is not a good practice to combat bacterial contamination during industrial-scale ethanol production. Even if lowering the medium pH reduces growth and metabolism of contaminating bacteria significantly, it also reduces the efficiency of yeast to convert sugars to ethanol, which ultimately results in reduced ethanol yield. By setting the mash with a higher content of dissolved solids of 30% (wt/vol) at the optimum pH for ethanol production by yeast of 5.0 to 5.5, the bacteria can be controlled efficiently and maximum ethanol production by the yeast can be achieved.

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